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GATA3 Targets Semaphorin 3B in Mammary Epithelial Cells to Suppress Breast Cancer Progression and Metastasis

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Abstract

Semaphorin 3B (SEMA3B) is a secreted axonal guidance molecule that is expressed during development and throughout adulthood. Recently, *SEMA3B* has emerged as a tumor suppressor in non-neuronal cells. Here we show that *SEMA3B* is a direct target of GATA3 transcriptional activity. GATA3 is a key transcription factor that regulates genes involved in mammary luminal cell differentiation and tumor suppression. We show that GATA3 relies on *SEMA3B* for suppression of tumor growth. Loss of *SEMA3B* renders GATA3 inactive and promotes aggressive breast cancer development. Overexpression of *SEMA3B* in cells lacking GATA3 induces a GATA3-like phenotype and higher levels of *SEMA3B* are associated with better cancer patient prognosis. Moreover, *SEMA3B* interferes with activation of LIM kinases (LIMK1 and LIMK2) to abrogate breast cancer progression. Our data provide new insights into the role of *SEMA3B* in mammary gland and provides a new branch of GATA3 signaling that is pivotal for inhibition of breast cancer progression and metastasis.

Keywords

GATA3; Semaphorin 3B; Breast Cancer; Metastasis

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Authors' Contributions:

P.S., C.-Y.W. and Z.W. designed the project; P.S., C.-Y.W., C. H., H.G.V., A. R., and Y.Y. performed the experiments; J.C. contributed material; P.S., C.-Y.W. and Z.W. analyzed and interpreted the data; M.-D.L provided supervision; P.S., C.-Y.W. and Z.W. wrote the paper.

Conflict of Interest

Authors declare no conflict of interest.

Introduction

Semaphorins function as short-range axonal guidance molecules(1). There are eight classes of semaphorins, and class-3 semaphorins are the only secreted group in vertebrates(2). Within class-3, there are seven semaphorin members that belong to semaphorin/collapsin family and exert their cellular effects through binding to neuropilins and plexin receptor family members(2, 3). SEMA3B is a secreted molecule that contains a highly conserved Sema domain in the amino terminus. Interaction between semaphorins and their receptors leads to changes in cytoskeletal architecture and cellular motility through influencing the activation state of various Rho GTPases in a targeted cell(4–9). Semaphorins along with other axonal guidance molecules function as environmental cues to allow proper innervation of neurons by either permitting or inhibiting their growth, thereby guiding neurites migration along a correct path during nervous system development.

While SEMA3B was initially discovered as an inhibitory axonal guidance molecule, recent findings suggest that SEMA3B also functions as a tumor suppressor in lung, renal, gastric and potentially breast tissues(10–14). In the mammary gland, SEMA3B is present in both luminal and basal epithelial cells(3). However, the importance of *SEMA3B* in maintenance of mammary epithelial cell homeostasis remains unclear.

We and others previously showed that GATA3 is essential for driving mammary epithelial cell differentiation and maintaining mammary gland homeostasis(15–18). GATA3 belongs to GATA family of transcription factors that play fundamental roles as master regulators of cellular differentiation and homeostasis in various tissues(15, 19–22). The GATA family is comprised of six members that are expressed in a tissue-specific manner(23). All GATA family members contain two transactivation domains in the amino terminus and two conserved zinc finger domains at the carboxyl terminus. In addition to their structural similarities, GATA family members recognize and bind to a consensus DNA sequence (A/T)GATA(A/G) to regulate downstream target genes(24).

Loss of or mutations in GATA3 results in development of aggressive breast cancer(25–30). Indeed, *GATA3* is one of the top three mutated genes in breast cancer patients(16), emphasizing its importance in tumor development. Therefore identifying new GATA3 downstream target genes provides valuable information in discovering new cancer biomarkers and potential therapeutic strategies for prevention of breast cancer.

In the present study, we establish *SEMA3B* as a new GATA3 downstream target gene that is indispensible for GATA3 tumor suppressive activity. In the absence of GATA3, elevated *SEMA3B* levels independently interfere with aggressive tumor growth and higher *SEMA3B* levels is associated with better breast cancer patient prognosis. We also show that SEMA3B interferes with activation of LIM kinases (LIMK1 and LIMK2) that are known Rho GTPases downstream targets. Inhibition of LIMK1/2 activation provides a molecular mechanism for inhibition of breast cancer progression via SEMA3B. Our findings highlight the importance of *SEMA3B* as a GATA3 downstream target gene and provide a new mechanism for driving tumor suppression in mammary epithelial cells.

Results

In silico Analysis of Breast Cancer Patient Samples Demonstrates a Correlation Between Expression of *GATA3* and *SEMA3B*

We initially performed *in silico* gene expression analysis by utilizing The Cancer Genome Atlas (TCGA) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) databases to determine the global gene expression alterations that correlate with changes in *GATA3* levels. Gene expression analysis of 597 breast cancer samples as well as RNAseq analysis of 1215 tumor samples from TCGA database indicated a strong correlation between loss of *GATA3* and downregulation of *SEMA3B* (Fig. 1a and Supplementary Figure 1 and 2). To demonstrate the validity of our analysis, we also included the expression analysis of a pro-tumor gene *MYC* that is upregulated in the absence of *GATA3* expression (Fig. 1a). TCGA database analysis using Regulome Explorer (http://www.cancerregulome.org/) indicated that SEMA3B resides in the same protein network as GATA3 and several other key proteins such as BCL2, ESR1, CCNB1 and AR (Fig. 1b) and suggested a possible link between GATA3 levels and SEMA3B expression during breast cancer development.

To investigate a potential role for SEMA3B in breast cancer development, we performed analysis using a public microarray dataset (GSE9014). The data suggest that *SEMA3B* is significantly downregulated in the samples from patients with invasive breast carcinoma (Fig. 1c). Similarly, comparison of *SEMA3B* expression in normal versus triple negative breast cancer samples using the TCGA database also indicated significant *SEMA3B* downregulation in triple negative breast cancer patients (Fig. 1d). We also examined *SEMA3B* levels across PAM50 breast cancer subtypes using METABRIC database (Fig. 1e) showing that *SEMA3B* levels remain high in luminal cancers. However, *SEMA3B* levels decreases significantly in basal subtypes (Fig. 1e). This analysis in PAM50 subtypes reinforces the importance of SEMA3B and suggests a correlation between the loss of GATA3 and SEMA3B levels during aggressive breast cancer formation. Moreover, there was a correlation between *SEMA3B* and breast cancer progression, with decreasing expression levels of *SEMA3B* as the tumors progress from stage I to stage IV (Suppl. Fig. 3). We observed a similar trend in downregulation of *SEMA3B* expression when we compared low-grade tumors to higher-grade tumors in the affected patients (Suppl. Fig. 4). Finally, analysis of the van't Veer breast cancer database for breast cancer survival outcome(31) indicated a poorer prognosis for patients with lowered *SEMA3B* expression (Fig. 1f). Collectively, the *in silico* analysis of breast cancer patient samples provides clues to a potential role for SEMA3B during breast cancer development and suggests an interplay between GATA3 and SEMA3B.

GATA3 Directly Controls SEMA3B Expression

To decipher whether GATA3 directly or indirectly controls SEMA3B levels, we performed *in silico* analysis using the ENCODE ChIP-Seq database. We searched for potential promoter sequences that contain a GATA3 binding site and found that the *SEMA3B* promoter is a potential target of GATA3 (Suppl. Fig. 5). We then performed chromatin immunoprecipitation analysis (ChIP) in three different transiently transfected mammary cell

lines, EpH4.9, T47D and MDA-MB-231 to examine whether GATA3 directly targets *SEMA3B* promoter. We found that in all these cell lines, GATA3 binds to the *SEMA3B* promoter (Fig. 2a). Next, we overexpressed *GATA3* in MDA-MB-231 cells and monitored alterations in *SEMA3B* expression levels. The control MDA-MB-231 cells exhibited minimal *GATA3* and *SEMA3B* expression. However, overexpression of *GATA3* resulted in significant upregulation of *SEMA3B* in the MDA-MB-231 cells (Fig. 2b and 2c). Additionally, immunostaining analysis demonstrated higher SEMA3B levels in cells overexpressing GATA3 when compared to control cells (Fig. 2d).

To study the GATA3 signaling pathway, we examined the relationship between GATA3 and SEMA3B in NMuMG cells that exhibit high levels of endogenous GATA3 expression. Knockdown of endogenous *GATA3* levels using a specific siRNA resulted in lowering *SEMA3B* expression levels, suggesting a correlation between GATA3 and SEMA3B levels in mammary cells (Fig. 2e). Taken together, these data indicate that in mammary epithelial cells, GATA3 directly binds to *SEMA3B* promoter and positively regulates *SEMA3B* expression.

GATA3 Relies on Intact SEMA3B Expression to Inhibit Cellular Migration and Proliferation

To investigate the interplay between GATA3 and SEMA3B, we generated multiple MDA-MB-231 stable cell lines that either expressed the control shRNA MDA-MB-GATA3-PLKO (MDA-GATA3) or shRNA targeting *SEMA3B* (MDA-GATA3-SEMA3B^{KD}). In 2D culture, cells overexpressing GATA3 lost their spindle-like morphology and became more cuboidal (luminal epithelial-like) (Fig. 3a), as we described previously(32). Moreover, the MDA-GATA3 cells formed tight colonies as opposed to the more dispersed colonies of control cells (Fig. 3a). Intriguingly, in the absence of *SEMA3B*, the GATA3 overexpressing cells reverted back to spindle-like morphology and resembled MDA-MB-231 (control) colonies that lacked GATA3 expression (Fig. 3a).

We also examined the effect of *SEMA3B* overexpression independent of GATA3 signaling in MDA-MB-231 cells. Since SEMA3B is downstream of GATA3, we were interested to determine whether SEMA3B could mimic GATA3 activity. Therefore, we generated MDA-MB-231 cells that either contained the control plasmid, MDA-MB-231-pEIZ (Control) or overexpressed *SEMA3B*, MDA-MB-231-SEMA3B (MDA-SEMA3B). Upon overexpression SEMA3B alone, MDA-MB-231 cells mimicked GATA3 overexpressing cells and formed tight cuboidal colonies (Fig. 3a). In three-dimensional (3D) Matrigel cultures, MDA-MB-231 (control) cells formed invasive colonies rich in invadopodia. However, upon overexpression of GATA3, the cells formed dense spherical colonies with minimal invadopodia (Fig. 3b). Upon overexpression of SEMA3B alone, MDA-MB-231 cells formed less invasive GATA3-like colonies, recapitulating the GATA3 phenotype (Fig. 3b). Interestingly, knockdown of *SEMA3B* even in the presence of GATA3 expression rescued the invasive phenotype and the colonies resembled the parental MDA-MB-231 cells (Fig. 3b). To show that these observations are not exclusive to MDA-MB-231 cells, we examined the effect of *SEMA3B* knockdown in EpH4.9 mammary epithelial cells. EpH4.9 cells have detectable endogenous GATA3 and SEMA3B levels and in 3D Matrigel cultures form dense colonies that exhibit minimal invadopodia. Again, knockdown of *SEMA3B* in EpH4.9 cells

resulted in formation of aggressively invasive colonies when compared to control (Suppl. Fig. 6). These observations indicate that SEMA3B plays a significant role in disrupting cellular invasiveness in mammary epithelial cell. Furthermore, GATA3 signaling requires intact SEMA3B to disrupt the invasive phenotype observed in MDA-MB-231 cells.

Next we examined the effect of *SEMA3B* on expression levels of epithelial to mesenchymal transition (EMT)-associated genes. Since SEMA3B inhibits cellular invasiveness and reduction in SEMA3B levels forms invasive colonies it is possible the SEMA3B could modulate EMT-associated genes. qPCR analysis in control and MDA-SEMA3B cells indicated that SEMA3B expression leads to reduction of the EMT genes *Vimentin*, *Fibronectin*, *Snail* and *N-Cadherin* expression levels (Fig. 3c), while, SEMA3B increasing *E-cadherin* expression (Fig. 3c). These results suggest that SEMA3B increases cell-cell interaction by lowering EMT genes and increasing E-cadherin levels.

To examine the effect of GATA3/SEMA3B signaling on MDA-MB-231 cell motility, we performed a scratch assay. MDA-MB-231 (control) cells were able to close the generated gap within 24 hours (Fig. 3d). However, MDA-MB-231 cells expressing either GATA3 or SEMA3B failed to fill the gap within the same time span (Fig. 3d). The observation suggests that GATA3 and SEMA3B interfere with cellular migration in MDA-MB-231 cells. Interestingly, MDA-GATA3-SEMA3B^{KD} cells closed the generated gap, similar to MDA-MB-231 parental cells, within 24 hours (Fig. 3d). These data suggest that GATA3 signaling relies on the presence of SEMA3B to inhibit cellular migration.

Next, we examined the effect of GATA3 and SEMA3B interplay in cellular proliferation using a colony formation assay in 2D cultures. Overexpression of SEMA3B resulted in formation of fewer colonies when compared to control (Fig. 3e). Inhibition of cellular proliferation and colony formation in cells expressing SEMA3B mirrored MDA-GATA3 cells. However, removal of SEMA3B in cells with intact GATA3 signaling restored cellular proliferation and colony formation (Fig. 3e; Suppl. Fig. 7). We observed similar behavior in mammary MCF7 tumor cells. Expression of SEMA3B significantly inhibited colony formation when compared to control. However, knockdown of *SEMA3B* even in presence of GATA3, rescued proliferative phenotype in MCF7 tumor cells (Suppl. Fig. 8).

Taken together, our *in vitro* data suggest that the presence of SEMA3B is pivotal for effective GATA3 signaling and intact GATA3/SEMA3B signaling axis is essential for inhibition of abnormal cellular migration and proliferation.

Tumor Suppressive Activity Exerted by GATA3 Requires Intact SEMA3B Expression

To determine the necessity of SEMA3B in GATA3 tumor suppressive activity, we orthotopically transplanted the MDA-MB-231 stable cell lines via intra-mammary gland injection into 8-week-old female nude mice. To examine the role of SEMA3B independent of GATA3 signaling in tumor growth, we compared control (SEMA3B-low) to MDA-SEMA3B (SEMA3B-high) cell transplants. Overexpression of SEMA3B reduced tumor growth and resulted in the formation of significantly smaller tumors when compared to control (Fig. 4a). To further elaborate on the importance of SEMA3B as a signal transducer molecule downstream of GATA3, we also transplanted tumor cells expressing GATA3 with

or without the intact SEMA3B molecule. Tumor cells overexpressing GATA3 demonstrated slower tumor growth and smaller tumor size(17, 25, 26, 29, 31, 32). In our transplant experiments, analysis of tumor sizes indicated that MDA-GATA3-SEMA3B^{KD} cells give rise to significantly larger tumors when compared to tumors from MDA-GATA3 cells. These results suggest the need for presence of SEMA3B for proper GATA3 tumor suppressive activity (Fig. 4b). Thus, *in vivo* SEMA3B mimics GATA3 activity in hindering tumor growth. We also performed Ki67 staining to assess the cellular proliferation status of the tumor cells in our samples. Cells in control tumors stained strongly for Ki67, while MDA-GATA3 or MDA-SEMA3B tumors had many fewer Ki67 positive cells (Fig. 4c). Sections from MDA-GATA3-Sema3B^{KD} tumors had more intense Ki67 staining than MDA-GATA3 or MDA-SEMA3B tumor sections. Knockdown of SEMA3B in tumor cells expressing GATA3 restored the proliferative ability of tumor cells and support the observations that GATA3 relies on SEMA3B to transduce its tumor suppressive activity (Fig. 4c).

SEMA3B Decreases Tumor Metastasis

The most significant factor in breast cancer aggressiveness is its propensity to metastasize. Therefore, we asked whether SEMA3B regulated metastasis of MBA-MB-231 tumors. We investigated tumor cell metastasis to inguinal lymph nodes in the orthotopically transplanted mice. Mice with control MDA-MB-231 or MDA-SEMA3B^{KD} tumors had significantly larger lymph nodes, larger lymph node metastatic colonies and higher number of metastatic cells in the lymph nodes than the mice with tumors expressing GATA3 or SEMA3B (Fig 4d, e). Thus our data suggest that SEMA3B can interfere with tumor metastasis.

Previous studies have indicated that semaphorins can regulate cell migration through Rho family of small GTPases (4–9). Recent reports indicate that LIM kinases (LIMK1 and LIMK2) that are downstream targets of Rho GTPases, play a role in enhancing breast cancer progression and metastasis(33–37). Since SEMA3B interferes with breast cancer progression, we asked whether SEMA3B influences LIMK1 and LIMK2 activity. Functional activation of LIM kinases requires phosphorylation at LIMK1 (threonine 508) and LIMK2 (threonine 505) (38, 39). We analyzed phospho-LIMK1/2 in control MDA-MB-231 and MDA-SEMA3B cells. Western blot analysis indicated that SEMA3B reduces phospho-LIMK1/2 levels when compared to control (Fig. 4f). Reduction of phopho-LIMK1/2 suggests one potential mechanism whereby SEMA3B could inhibit breast cancer progression and metastasis.

Collectively, our experiments support the importance of SEMA3B as a component of GATA3 signaling axis in mammary cells and provide evidence that SEMA3B can inhibit tumor growth as well as tumor metastasis downstream of GATA3 signaling.

Discussion

SEMA3B was originally identified as a neurite guidance molecule during nervous system development. However, recently, alternative roles for SEMA3B in non-neuronal adult cells have been described, highlighting the diverse functionality of developmental genes in an adult organism. In this study we have shown that SEMA3B is a direct target of he transcription factor GATA3 and that loss of *SEMA3B* correlates with aggressive mammary

tumor development and progression. GATA3 plays a fundamental role in maintaining mammary gland homeostasis by promoting luminal epithelial cell differentiation and also acts as a tumor suppressor by transcriptional regulation to influence the expression of a plethora of genes including pro-tumor factors (32). Our tumor transplant data indicates that overexpression of SEMA3B interferes with tumor metastasis similar to tumors expressing GATA3. By experimental analyses we showed that downregulation of SEMA3B leads to cellular proliferation, tumor growth and enhanced tumor invasiveness, even in presence of intact GATA3. We also show that SEMA3B inhibits tumor invasiveness by downregulating EMT-associated genes and increasing E-cadherin levels. Additionally, we found that SEMA3B, inhibits phosphorylation and subsequent activation of LIMK1/2. LIMK1/2 have been shown to promote breast cancer progression, therefore inhibition of LIMK1/2 is one potential mechanism for SEMA3B to exert inhibitory effects during breast cancer progression.

SEMA3B is a secreted molecule and it is important to consider its tumor suppressive influence beyond its potential autocrine functions. It is likely that other SEMA3B producing cells in the tumor microenvironment could influence the behavior of tumor cells. Loss of SEMA3B expression in cells of tumor microenvironment as well as tumor cells themselves could result in formation of aggressive breast cancer. To that extent, we hypothesize that the GATA3/SEMA3B signaling presents an important regulatory pathway that directs anti-tumor activity in the mammary gland.

The fact that a neuronal specific gene such as *SEMA3B* could influence mammary gland homeostasis is intriguing, building on other examples of such diversity. For example SLITs and their receptor ROBO were originally identified as axonal guidance signaling molecules during development(40, 41), but have been shown to play alternative roles in mammary gland branching(42), mammary stem cell self-renewal(43) and breast cancer development(44).

Recently, *GATA3* has emerged as one of top three genes associated with breast cancer development. Thus, uncovering new GATA3 signaling axes could help to better understand breast cancer development. Previously, we have shown that tumor suppressive activity of GATA3 is partially mediated through direct upregulation of *miR-29b*(32). *miR-29b* functions as a signal transducer of GATA3 and directs many anti-tumor activities via targeting a collection of genes that are involved in angiogenesis and remodeling the tumor microenvironment. Preliminary studies indicate that induction of SEMA3B via GATA3 is independent *miR-29b*. Our findings suggest that GATA3 induces multiple anti-tumor pathways to inhibit mammary tumor development. Redundant regulatory pathways could provide a safeguard for proper cellular functioning. The GATA3/SEMA3B signaling could function in parallel with other pathways such as GATA3/*miR-29b* to ensure proper cellular function in mammary epithelial cells.

There are several possibilities as to how lack of SEMA3B expression could override GATA3 activity. SEMA3B forms a complex with neuropilins and plexins on the cell surface. VEGF-A also binds to neuropilins and this complex promotes cellular migration(45, 46). In tumor cells, upregulation of SEMA3B via GATA3 could push the competition for neuropilin

binding to favor SEMA3B. In turn, elevated SEMA3B levels could outcompete VEGF-A for binding to neuropilins, thereby disrupting tumor cell migration. Additionally, since SEMA3B can inhibit phosphatidylinositol 3-kinase (PI3K)/Akt(47), upregulation of SEMA3B by GATA3 could result in downregulation of PI3K/Akt activity and lead to inhibition of cellular proliferation and tumor growth.

Collectively, our data shed new insights into better understanding of GATA3 pathway in the mammary gland. We identified SEMA3B as a direct target of the GATA3 transcription factor and highlighted its importance as a signal transducer molecule. Loss of SEMA3B overrides GATA3's instructive ability to function as a tumor suppressor and designates SEMA3B as an integral part of GATA3 pathway in mammary epithelial cells. Our findings introduce SEMA3B as a molecular target that could potentially provide new therapeutic strategies against aggressive breast cancer.

Methods

Cell Culture and siRNA Constructs

T47D tumor cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% FBS, insulin and antibiotics. MDA-MB-231 cells (ATCC) were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics. EpH4.9 cells were cultured in DME-H21 medium supplemented with 5% fetal bovine serum (FBS), insulin (5 μg/ml) and antibiotics. 3D Matrigel culture assays were performed as described(32). The lentiviral SEMA3B shRNA constructs were generous gift from Dr. Luca Tamagnone. The purchased siRNA sequences were as follows: ON-TARGETplus GATA3 siRNA (Dharmacon). SEMA3B siRNA (Santa Cruz Biotechnology INC.).

Immunostaining, Histology and Antibodies

Immunofluorescence—Cells were cultured on coverslips prior to staining. Cells were washed with cold PBS and fixed with 4% paraformaldehyde for 20 min. Cells were washed with cold PBS and blocked with PBS, 5% goat serum and 0.25% Triton X-100 for at least 1 hour at room temperature. Coverslips were incubated with primary antibody diluted in PBS plus 5% goat serum overnight at 4°C. Coverslips were washed with PBS and incubated with secondary antibody for 1 hour at room temperature then washed with PBS and mounted with Vectashield mounting medium with DAPI (Vector Laboratories).

Immunohistochemistry—Tissues were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Tissues were sectioned into 5–7 μm sections and a standard protocol for hematoxylin and eosin staining was used for histological analysis. Immunostaining was performed using the sodium citrate protocol for antigen retrieval as previously described (15). Images were captured using Nikon C1si confocal microscope.

Antibodies—Actin-HRP (cs-47778, Santa Cruz Biotechnology), Alexa Fluor 546 goat anti-mouse (A-11008, ThermoFisher Scientific), Alexa Fluor 488 goat anti-rabbit (A-11030, ThermoFisher Scientific), GATA3 (AF2605, R&D Systems, Ki67 (ab15580, Abcam), Sema3B (sc-21204-R, Santa Cruz Biotechnology). Phoshop-LIMK1 (Thr508)/LIMK2

(Thr505) (3841T, Cell Signaling Technology). LIMK1 (VMA00361KT, Bio-Rad Laboratories Inc.). LIMK2 (8C11) (3845, Cell Signaling Technology).

Bioinformatics and Computational Analysis

SEMA3B expression analysis in human breast cancer and normal tissues was performed using the Oncomine database (www.oncomine.org). Statistical analysis was performed via Oncomine default algorithms(48). The TCGA data set obtained from Oncomine is embedded in the TCGA database (https://tcga-data.nci.nih.gov/tcga)(49). Heatmaps were generated from breast cancer TCGA database. The GSE9014 dataset obtained from Oncomine is embedded in NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo)(50) for SEMA3B expression analysis.

Kaplan–Meier log-rank tests were performed using default parameters in SurvExpress(51). Patient data was segregated into low or high SEMA3B expressing groups at the median expression value in all cases.

Promoter Analysis and Chromatin Immunoprecipitation Assay

SEMA3B promoter analysis was performed using the ENCODE ChIP-Seq data set. Primer sequences for ChIP analysis: Set #1, 5′-AGACAGGTATGACCGTGACC-3′ (forward) and 5′-AGCTGTCTTGTGCTTGGGAAT-3′ (reverse); Set #2, 5′-CAGACCTCATGGGACGAGAC-3′ (forward) and 5′-TGGCTAGCTGTCTTGTGCTT-3′ (reverse). GATA3 and SEMA3B chromatin immunoprecipitation analysis was performed using the ChIP-IT High-Sensitivity Kit as per manufacturer's protocol (Active Motif).

Scratch Assay

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After 24 hours, a monolayer of cells was scratched by a 200-μL pipette tip. Cell migration was quantified by counting the number of cells that had migrated to the scratched area within 24 hours.

Colony Formation Assay

Cells were seeded in 6-well plates at a density of 1×10^3 cells/well and cultured for 14 days. Cells were fixed using methanol and stained with Giemsa solution and colonies were counted.

Animal Studies

5×10^5 cells in 10 μl of 1:1 DMEM/Matrigel (BD Biosciences) were injected in the fat pads of female 8-week-old nude mice (Simonsen Laboratories). Once palpable, tumors were measured twice weekly and the tumor volumes were determined as follows: V = 0.52 × (length) 2 × width. All mouse procedures were approved by University of California, San Francisco, Institutional Animal Care and Use Committee.

Statistical Analysis

Statistical analysis was conducted using Prism 7 software (Graph Pad Software, Inc.). Statistical significance between two groups was calculated using Student's t test, A one-way

analysis of variance (ANOVA) was performed to determine two or more group differences with Bonferroni test and P values < 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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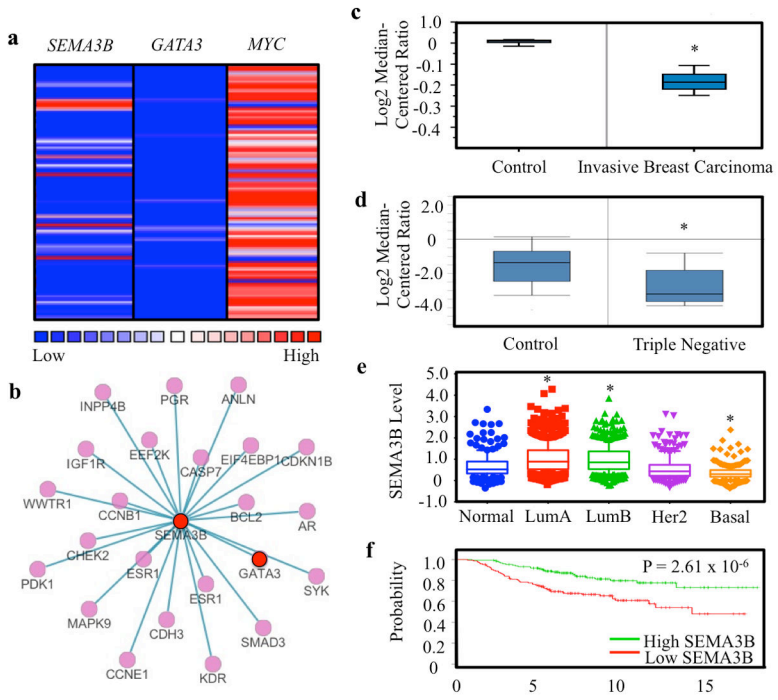

Figure 1.

In silico analysis of breast cancer databases. **(a)** Analysis of TCGA database. Heat map indicating gene expression analysis for *GATA3*, *SEMA3B* and *MYC* in multiple breast cancer samples. (N = 597) **(b)** Analysis of TCGA database via Regulome explorer. Data present a collection of proteins including GATA3 that may reside in the same protein network as SEMA3B. **(c)** Analysis of *SEMA3B* expression via the GSE9014 database. *SEMA3B* expression is significantly lowered in the invasive breast carcinoma samples. (Normal: N = 6, Invasive breast carcinoma: N = 53, P = 1.03E-21) **(d)** TCGA analysis indicating significantly lowered *SEMA3B* expression in triple negative breast cancer samples. (Normal: N = 244, Triple negative: N = 49, P = 4.06E-9) **(e)** Analysis of *SEMA3B* expression in PAM50 subtypes using the METABRIC database. Luminal breast cancers possess higher levels of *SEMA3B* expression than the basal subtype, P = <0.0001, Normal: N = 202, Luminal A: N = 721, Luminal B: N = 492. HER2: N = 240, Basal: N = 331) (f) Analysis of breast cancer patient survival (31). Tumor samples exhibiting lower *SEMA3B* expression show poorer patient prognosis. (Concordance index = 62.3, Log-rank equal curves P = 1.6E-4, R^ 2 = 0.005/0.941, Risk groups hazard ratio = 2.4, P = 2.61E-6)

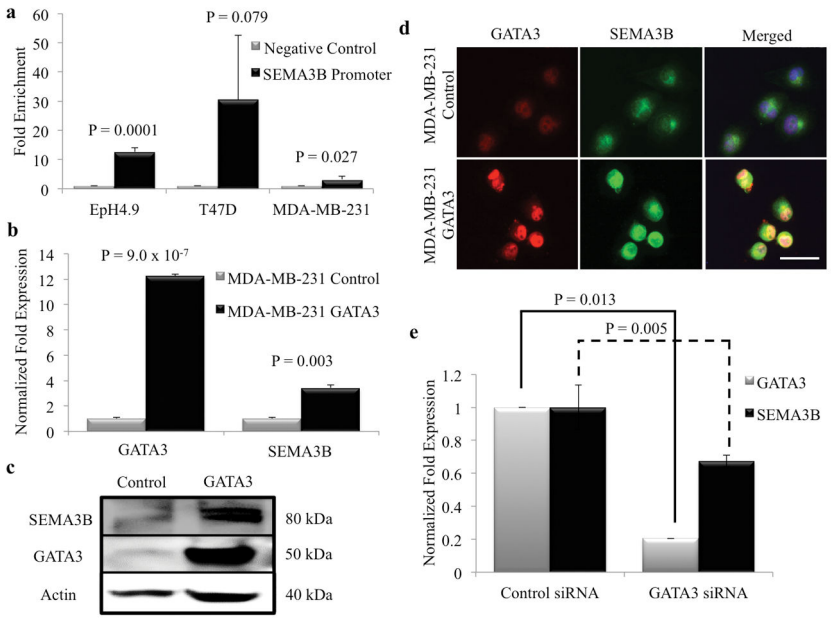

Figure 2.

GATA3 directly controls SEMA3B expression. **(a)** ChIP analysis indicating GATA3 transcription factor binding to *SEMA3B* promoter in EpH4.9, T47D and MDA-MB-231 cells. **(b)** qPCR analysis of *GATA3* and *SEMA3B* expression in MDA-MB-231 cells, N = 6. Overexpression of *GATA3* in MDA-MB-231 cells results in elevation of *SEMA3B* levels. **(c)** Western blot analysis of MDA-MB-231 cell lysate. Overexpression of GATA3 enhances SEMA3B expression. **(d)** Immunostaining of MDA-MB-231 (control) and GATA3 overexpressing MDA-MB-231 cells. Overexpression of *GATA3* upregulates *SEMA3B* levels. Bar 50 μm. **(e)** qPCR analysis of *SEMA3B* in NMuMG cells, N = 2. Lowering *GATA3* expression via siRNA results in downregulation of *SEMA3B* levels.

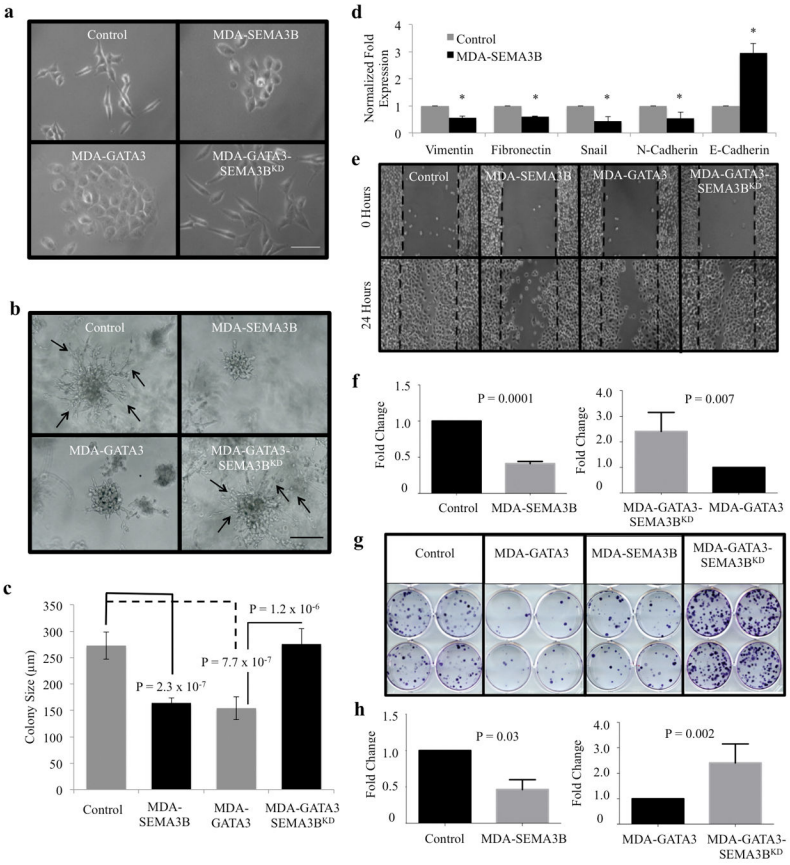

Figure 3.

Inhibition of cellular migration and proliferation by GATA3 relies on presence of SEMA3B. **(a)** Bright-field images of MDA-MB-231 stable cell lines in 2D cell cultures. Bar, 50 µm. **(b)** Phase-contrast images of MDA-MB-231 stable cell lines in 3D cultures. Arrows indicate the presence of invadopodia moving outward from the colony. Bar, 200 µm. (c) Quantification of 3D Matrigel culture colonies. (N = 7) (d) qRT-PCR analysis of EMT-associated genes. RNA samples from MDA-MB-231 control and MDA-SEMA3B cells were collected. qRT-PCR analysis indicates that SEMA3B lowers EMT gene levels. SEMA3B enhanced E-cadherin levels compared to control. **(e)** Scratch assay analysis indicating difference in cellular migration in MDA-MB-231 stable cell lines. N = 6 **(f)** Quantification of cell infiltration of gaps between the two invading cell fronts. The gap was measured using ImageJ software. Y-axis indicates experimental group/control group. (N = 6). (g) Colony formation assay. MDA-MB-231 stable cell lines were cultured in 6-well plates to allow colony growth. Colonies were stained with crystal violet and counted. N = 12. (h) Graph representing the quantification of Giemsa stained colonies. (N = 12).

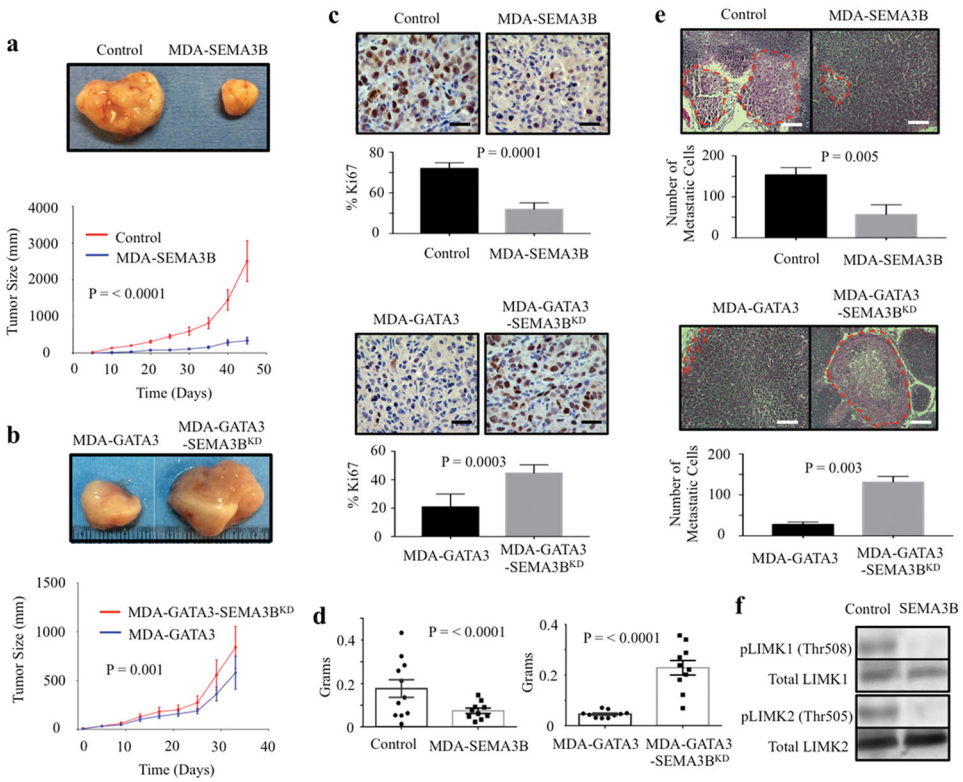

Figure 4.

Loss of SEMA3B disrupts GATA3 tumor suppressive activity. **(a)** MDA-SEMA3B and **(b)** MDA-GATA3-SEMA3B^{KD} stable cell lines where transplanted in 8-week-old female nude mice via orthotopic injection. Color photographs show a representative set of gross tumors from each transplanted group. Graphs indicates tumor measurement during the course of experiment, N = 10 for each group. **(c)** Immunostaining and quantification analysis for Ki67 expression in the tumor sections (N = 6). Bar, 100 μm. **(d)** Analysis of inguinal lymph node weight from transplanted mice, N = 10 for each group. **(e)** H&E analysis of lymph node sections indicating tumor metastasis. For each transplanted group, metastatic tumor cells to the lymph node was quantified (N = 6). Bar = 200 μm. **(f)** Western blot analysis indicating phosphorylation status of LIMK1 and LIMK2 in control and MDA-SEMA3B cell extracts. Total LIMK1 and LIMK2 indicate equal loading of samples. Protein bands 72 kDa.